Chickpea Defensin Gene Family: Promising Candidates for Resistance Against Soil-Borne Chickpea Fungal Pathogens

Article *in* Journal of Plant Growth Regulation · October 2022

DOI: 10.1007/s00344-022-10811-1

CITATIONS
0

READS
257

10 authors, including:

Rahul Nitnavare
Rothamsted Research
31 PUBLICATIONS **454** CITATIONS
SEE PROFILE

Richa Yeshvekar
University of Leeds
8 PUBLICATIONS **204** CITATIONS
SEE PROFILE

Joorie Bhattacharya
International Crops Research Institute for the Semi Arid Tropics
18 PUBLICATIONS **97** CITATIONS
SEE PROFILE

Vidhi Sapara
International Crops Research Institute for Semi Arid Tropics
1 PUBLICATION **0** CITATIONS
SEE PROFILE

Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-022-10811-1

Chickpea *Defensin* Gene Family: Promising Candidates for Resistance Against Soil-Borne Chickpea Fungal Pathogens

Rahul B. Nitnavare[1,2] · Arunima Pothana[3] · Richa K. Yeshvekar[4] · Joorie Bhattacharya[3,5] · Vidhi Sapara[3,5] · Palakolanu Sudhakar Reddy[3] · Yogendra Ramtirtha[6] · Avijit Tarafdar[3] · Mamta Sharma[3] · Pooja Bhatnagar-Mathur[3,7]

Received: 19 February 2022 / Accepted: 15 August 2022

Abstract

Defensins are broad-spectrum antimicrobial peptides that play an important role in providing innate immunity to various biotic stresses in plants. We identified and characterized 22 defensin (DEF) and defensin-like (DEFL) genes in chickpea (*Cicer arientinum*) based on their structures, expression, chromosomal localization, conserved motifs, and *cis*-regulatory elements. The localization of *DEF* and *DEFL* genes in chickpea genome revealed the presence of at least two clusters that are likely evolved through local gene duplications. Genotype-specific responses of several *CaDEF* and *CaDEFL* genes in fungal bioassays suggested their involvement in defense against fungal pathogens such as hemi-biotrophic *F. oxysporum* f. sp. *ciceris* and dry root rot causing necrotrophic *R. bataticola*. Molecular docking studies revealed interactions of CaDEFs with fungal plasma membrane components such as phosphatidylserine (PS) and glucosylceramide (GluCer) and their binding sites were identified. Our data will be useful to identify potential candidate genes and their role in host-plant resistance in chickpea, besides presenting opportunities for their potential for possible deployment in other crops.

Keywords Anti-fungal proteins (AFPs) · Chickpea · Defensins, dry root rot · Fusarium · Host pathogen interactions

Handling Editor: Padmanabh Dwivedi.

Rahul B. Nitnavare, Arunima Pothana and Richa K. Yeshvekar contributed equally for this article.

✉ Pooja Bhatnagar-Mathur
p.b.mathur@cgiar.org

1 Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, UK

2 Plant Sciences Department, Rothamsted Research, Harpenden AL5 2JQ, UK

3 International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana 502324, India

4 Centre for Plant Sciences, School of Biology, University of Leeds, Leeds LS2 9GT, UK

5 Department of Genetics, Osmania University, Hyderabad, Telangana 500007, India

6 Indian Institute for Science Education and Research, Pune, Maharashtra 411007, India

7 International Maize and Wheat Improvement Center (CIMMYT), Mexico-Veracruz, El Batán Km. 45, 56237 Mexico, Mexico

Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinating annual diploid legume with an estimated genome size of ~ 740 Mb (Jain et al. 2013; Varshney et al. 2013). In the semi-arid tropics, chickpea is consumed as a primary source of protein and while it is an important legume and is a member of the founder crop package, research pertaining to it is limited compared to the other founder crops (Jukanti et al. 2012). Fungal diseases account for almost 70% of the major crop diseases and chickpea production is affected by several fungal diseases such as Fusarium wilt, Botrytis grey mold, Dry root rot and Ascochyta blight (Islam 2008; Li et al. 2015). Various breeding strategies such as identification of Quantitative Trait Loci (QTL) using resistant sources and marker assisted selection (MAS) have been employed for developing chickpea cultivars with high level of resistance to diseases and pests. Nevertheless, to overcome difficulties associated with introgression of large QTL, it is important that candidate genes contributing to fungal disease resistance in this important legume crop are identified. Plants have been found to combat such fungal diseases with the help of antifungal proteins (AFPs). These AFPs are categorized according

Published online: 03 October 2022

to their structure and function and comprise of proteins such as defensins and defensin-like proteins, chitinases and chitinase-like proteins, peroxidases, and ribonucleases among many (Yan et al. 2015). While leguminous plants have no immune system, defensins form a part of the innate immunity (Wang et al. 2009; Lacerda et al. 2014). Plant defensins, a diverse family of antimicrobial peptides, play an important role in defense against pathogens by inhibiting their growth and infection (Yan et al. 2015). Defensins accumulate in high concentrations at the site of infection during initial stages to arrest the pathogen attack and are produced in moderate levels in rest of the plant as a part of induced systemic resistance. They have been reported to form pores in the pathogen plasma membrane and increase the Ca^{2+} uptake and K^{+} influx, thereby increasing the cell permeability which is similar to numerous other AFPs (Iqbal et al. 2019). Several defensin proteins and peptides have been isolated from legumes which confer antifungal activities (Mani- López et al. 2021). These properties of defensins have been utilized in transforming several plant defensins into various host plants to enhance disease resistance (Lacerda et al. 2014; Gao et al. 2000; Lay and Anderson 2005). Although diverse in their primary sequences, defensins are 45–54 amino acids in length with a characteristic N-terminal signal peptide, defensin-motif, and conserved cysteine residues (Iqbal et al. 2019). The three-dimensional structure of plant defensins comprises of a α-helix with triple stranded antiparallel β-sheets stabilized by four intra-molecular disulphide bridges (CSα/β) whose organizational patterns are well conserved. These structural features confer a wide range of biological properties to defensins, such as antifungal and antibacterial activities (Aerts et al. 2008), zinc tolerance (Mirouze et al. 2006), proteinase inhibitory activity (Wijaya et al. 2000), and α-amylase inhibitory activity.

A number of *defensin* (*DEF*) and *defensin-like* (*DEFL*) genes have been identified from model species like *A. thaliana*, *Medicago truncatula,* legumes such as *Pisum sativum, Phaseolus vulgaris* and crops plants such as *Brassica rapa, Vitis vinifera*, and *Solanum lycopersicum* (Mani- López et al. 2021). Nevertheless, the defensin gene family has not yet been explored in chickpea (*Cicer arietinum* L.) except for studies against *Ascochyta rabiei* (Pass.) Labr (Andam et al. 2020). Considering the relevance and potential of these antimicrobial peptides for resistance-associated mechanisms, our study explored *DEF* and *DEFL* genes in chickpea with detailed information on their structures, chromosomal localization, conserved motifs, cis-regulatory elements, and their expression, during pathogen attack against *F. oxysporum* f. sp. *ciceris* and *Rhizoctonia bataticola.*

Materials and Methods

Plant Material, Stress Imposition, and Growth Conditions

Two groups of chickpea genotypes were used in this study. The first group constituted of *Cicer arietinum* L. varieties, WR 315 and JG 62, reported to be highly resistant and susceptible to *Fusarium oxysporum* f. sp. *ciceris*, respectively (Sharma et al. 2010). The second group constituted of a wild chickpea species found to be resistant (*C. reticulatum;* ICC 17160), and cultivated *C. arietinum* varieties that are moderately resistant (ICC 005530), or susceptible (BG 212) to dry root rot caused by *Rhizoctonia bataticola* (*Macrophomina phaseolina*). For first group, seeds of JG 62 and WR 315 were surface sterilized using 2% sodium hypochlorite for 2 min, rinsed in sterile distilled water, and germinated for eight days in plastic pots containing sterilized sand. The 8-day old seedlings were carefully uprooted, and the roots were washed under running water to remove excess sand. Root tips, around 0.5 cm long were cut off to facilitate entry of *Fusarium oxysporum* f. sp. *ciceris* (Foc) Race1 into the roots. Roots of the seedlings were then dipped separately in the *F. oxysporum* inoculum @ 6.5×10^5 conidia ml^{-1} for 1–2 min to enable conidia to adhere to the roots. Inoculated seedlings were transplanted in pre-irrigated sterile vertisol and sand (3:1) in pots and incubated at 25 ± 3 °C. In the resistant genotype WR 315, no disease symptoms were observed until the end of experiment, but susceptible genotype JG 62 showed typical wilt symptoms like dropping of the leaves, flaccidity and vascular discoloration leading to complete mortality of the plant. Visible symptoms are seen 7 days after inoculation and complete mortality was observed after 10–12 days under controlled conditions. Inoculated seedlings were observed regularly for disease expression. Sampling was done at regular intervals of 24 h until 288 h after inoculation. Total genomic DNA from Foc infected root samples of both genotypes, WR 315 and JG 62 was isolated using PureLink Plant Total DNA Purification kit (Invitrogen, USA) followed by manufacturer's recommendation. To quantify the colonization of Foc within the chickpea root tissue, the real-time PCR was carried out as per the protocol of Sharma et al. (2014) and Tarafdar et al. (2018) using gene specific 18 s primer pairs using 10 ng of each DNA. The qRT-PCR was carried out with the amplification cycle of 95 °C for 4 min, and another 40 cycles of 95 °C for 10 s, and 62 °C for 30 s. To make a standard curve for quantification of Foc in root tissue, the tenfold diluted DNA of Foc 38 ranging from 10 ng to 0.01 pg were subjected to qPCR. The triplicate Ct values were taken for each sample as replications in every experiment and standard error was calculated.

For the second group, 7-day-old seedlings of *C. arietinum* varieties BG 212, ICC 05530, and *C. reticulatum* (ICC 17160), raised in plastic pots containing sterilized sand, were inoculated by dipping the roots in macerated culture of *R. bataticola* for 2 min.. A pathogenic isolate of *R. bataticola*, isolated from naturally infected chickpea plant at ICRISAT, Patancheru, was used throughout the experiments. Isolate was purified using mono-sclerotia and maintained on PDA slants at 5 °C in the refrigerator. Inoculum was mass multiplied on potato dextrose broth medium. *R. bataticola* was inoculated from actively growing fungal disc into a potato dextrose broth and incubated for 5 days at 28 °C. The fungal mat from this broth was macerated in 100 ml of sterilized, distilled water (SDW) which was then used as inoculum for dipping the roots (Sharma and Pande 2013). Inoculated seedlings were placed in a folded, moist blotting paper with shoot protruding outside and placed in trays followed by incubation at 35 ± 1 °C with a 12 h photoperiod. The seedlings were regularly moistened with sterile distilled water for 7 days following which disease severity was evaluated at regularly. In susceptible cultivars, the roots of the affected plant showed black lesions and discoloration is very prominent. Severity of the disease varies in moderate and susceptible cultivars). Blotter paper was adequately moistened every alternate day till final observation. Incubation conditions were 35 °C and 12 h of photoperiod. The resistant genotypes showed less disease severity in contrast to susceptible checks. Subsequently root samples were collected from infected and uninfected plants in duplicates at each time point until 168 h, and flash frozen in liquid nitrogen.

Identification and Phylogenetic Analysis of *CaDEF* Genes from Chickpea

Two different approaches were used for genome-wide identification of Defensins: (i) Firstly, Defensin (DEF) and Defensin-like (DEFL) protein sequences from plants belonging to Poaceae (*Hordeum vulgare, Zea mays, Cenchrus americanus, Oryza sativa, Sorghum bicolor, Triticum aestivum, Saccharum officinarum*), Fabaceae (*Medicago sativa, M. truncatula, Lotus corniculatus, Pisum sativum, Phaseolus vulgaris, Trigonella foenum, Vigna unguivulata*), Solanaceae (*Nicotiana sp., Atropa belladona, Capsicum annum, Lycopersicum esculentum, Solanum tuberosum*), and Brassicaceae (*A. thaliana, A. lyrata, A. halleri, Brassica sp., Raphanus sativus*) were used as queries to conduct extensive BLASTP searches against the NCBI database to identify potential chickpea defensins (CaDEFs). Unique sequences were selected manually and were analyzed using the Pfam and NCBI conserved domain database (CDD) to confirm these as members of defensin gene family. (ii) In the second approach, the same query sequences were used as input for Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) alignments. The alignment files were then used for making DEF HMM profile using module "hmmbuild". Further, all predicted protein sequences of chickpea were searched using this DEF HMM profile using module "hmmsearch" in the HMMER version V.3. with *e* value < 1e-05. The resulting sequences were further confirmed for the presence of defensin domains using PfamScan. Multiple sequence alignments of all DEF and DEFL proteins available from chickpea, *A. thaliana* and *M. truncatula* in the UniProtKB database were performed using ESPript (https://espript.ibcp.fr). Phylogenetic tree of all *CaDEF*, *AtDEF*, and *MtDEF* sequences was constructed using the Neighbor Joining (NJ) method in MEGA 7 software with bootstrap values from 1000 replicates indicated at each node. Similarly, an NJ-tree of CaDEFL, MtDEFL, and AtDEFL sequences was also constructed. An unrooted phylogenetic tree of the identified 16 CaDEFs and 6 CaDEFLs was constructed using similar method. The nomenclature used to assign gene names had two letters denoting the source organism, name of the gene family, group number and gene numeral (e.g., *CaDEF1.1, CaDEFL1*). The CDS, peptide lengths, and chromosomal locations were obtained from the NCBI database. Physicochemical characterization of proteins was performed by predicting the molecular weight (kDa), isoelectric point (pI) using the pI/Mw (http://web.expasy.org/compute_pi/) and PROTPARAM (http://web.expasy.org/protparam/) tools of EXPASY. Subcellular localization of CaDEF proteins was predicted using TargetP1.1 server (http://www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT tool (http://www.genscript.com/wolf-psort.html).

Structure and Organization

The intron–exon structures of *CaDEF* genes were determined by aligning the genomic and CDS sequences. The gene structure was represented by using Gene structure display server (GSDS) (http://gsds.cbi.pku.edu.cn/). Additionally, the MEME suite (http://meme-suite.org/tools/meme) was used for identification of novel un-gapped conserved motifs in CaDEF protein sequences.

In Silico Promoter Analysis

To elucidate putative *cis*-acting elements that may be responsible for *CaDEF* gene expression in response to different stresses, 1500 bp of the genomic DNA sequences upstream from the translational start site (ATG) of the each *CaDEF* gene were extracted from the NCBI database. The plantCARE (Lescot et al. 2002), PLACE (Higo et al. 1999), and PlantPAN (Chang et al. 2008) databases and existing literature were used to identify the *cis*-acting elements in the promoter regions.

Chromosomal Location and Synteny Analysis

The chromosomal location of *CaDEF*, *AtDEF*, and *MtDEF* genes was determined based upon information from the GenBank, NCBI. A representative image of chromosomal locations of *CaDEF* genes was created using MapChart version 2.30 (https://www.wur.nl/en/show/Mapchart-2.30.htm). To compare defensin genes from chickpea with those in other plant species such as *M. truncatula* and *A. thaliana*, BLASTN searches were conducted using the NCBI RefSeq database, with parameters; *e* value ≤ 0.01 and minimum percent identity = 70%. Genes identified on unplaced scaffolds were not used in the analysis. Ideograms were created using Circos (Krzywinski et al. 2009).

RNA Extraction and qRT-PCR Analysis

Total RNA from infected and uninfected chickpea root tissues were isolated using NucleoSpin RNA kit (Macherey–Nagel GmbH & Co. KG) according to manufacturer's instructions. The RNA (2 µg) was reverse-transcribed into the first-strand cDNA in 25 µl of reaction using MuLV Reverse Transcriptase (NEB). qRT-PCR primers were designed using Primer3 software (Untergasser et al. 2007) with GC content of 40–60%, Tm of 60–62 °C, and primer length of 20–22 nucleotides with an expected product size of 90–180 bp (Table S1). The Kappa Master Mix (2X) was used for gene quantification according to manufacturer's recommendations in the RealPlex (Eppendorf). qRT-PCR for each sample in duplicate was carried out in 96-well optical reaction plates in a total volume of 10 µl containing 0.4 µM of each primer, cDNA (1.0 µl), and 5 µl of Kappa master mix (2X) and nuclease-free water added up to 2.4 µl. The thermal cycles were as follows: 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Upon completion of qRT-PCR reactions, melting curves were generated to analyze the specificity of each gene by increasing the temperature from 60 to 95 °C. For internal calibration, *CAC* and *ABCT* genes were used (Reddy et al. 2016). The relative expression levels of *CaDEF* transcripts in resistant and susceptible genotypes in response to the disease progression were compared to their corresponding controls using the $2^{-\Delta\Delta Ct}$ method. Similarly, the expression profiles of *F. oxysporum* genes in wilt resistant as well as susceptible genotypes were studied, with *GAS1*, *SWI6*, *LAS21*, and *FKS1* used as the fungal reference gene. All experiments were carried out in two biological and two technical replications.

The genesBeta-1,3- 6 glucanosyltransferase (GAS1), ethanolaminephosphotransferase (LAS21), 1,3-beta-glucan 7 synthase (FKS1) and Switching-deficient transcription co-factor (SWI6) are involved in fungal cell wall synthesis. These proteins are induced to protect and boost the fungal growth under various stress conditions. Defensins are known to bind and interact with phospholipid components of the fungal cell membrane which then induces pore formation, membrane permeabilization, internalization and subsequently arrests the fungal growth (Poon et al. 2014). The expression profiles of these genes were conducted in infected root samples of different resistant and susceptible genotypes to determine the involvement of CaDEF's and CaDEFL's in defense mechanism.

Molecular Docking Analyses

To predict molecular interactions between CaDEF proteins (CaDEF1.1b, CaDEF2.5a, CaDEFL2, CaDEF3, CaDEF4, and CaDEF5) and ligands that are integral components of fungal plasma membrane, glucosylceramide (GluCer) and phosphatidylserine (PS), molecular docking was carried out in two steps, (1) three-dimensional (3D) structure prediction, and (2) structure-based search for ligand interactions. The 3D structure was modeled using homology modeling-based approach using the MODWEB server (https://modbase.compbio.ucsf.edu/modweb/) (Pieper et al. 2011), and the best model was manually selected. Docking study was conducted to study the interactions of CaDEF proteins with PS. The generated CaDEF models were superimposed upon PDB-ID 4CQK (that represents interaction between *Nicotiana alata* defensin1 and PS) and PDB-ID 2KSK (that represents interaction between sugarcane defensin-5 and GluCer) using topology-independent structure superimposition tool CLICK (Nguyen and Madhusudhan 2011) and with C-alpha and C-beta as representative atoms. 3-D co-ordinate file was obtained from PubChem, converted to PDBQT format using AutoDock Tools 4 (Morris et al. 2009) and independently docked with CaDEF models by using AutoDock Vina with default parameters (Trott and Olson 2009).

Results

Identification and Phylogenetic Analyses

The genome-wide identification of defensins from chickpea based upon homology as well as Hidden Markov Model (HMM) searches resulted in 22 unique proteins containing the PF00304 and PF01097 domains corresponding to the ɣ-thionin and defensin family, respectively. The identified proteins were classified in three groups based upon the presence of conserved domains in the peptides and their phylogenetic clustering. Phylogenetic analysis of peptide sequences corresponding to the identified 22 genes revealed their clustering in different groups that could be classified into three distinct clades (Fig. 1). Group CaDEF1 comprised of three members that shared 98% sequence similarity and were transcript

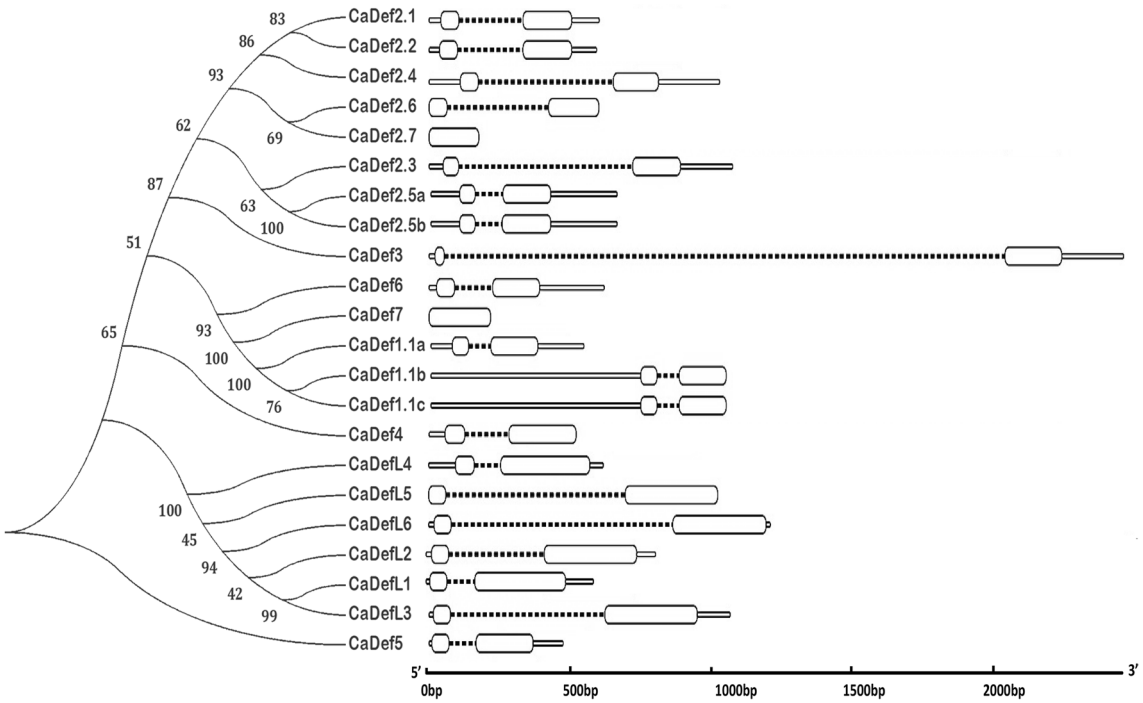

Fig. 1 Phylogenetic analysis of CaDEF and CaDEFL amino acid sequences. NJ-tree of 16 CaDEF and 6 CaDEFL proteins, and gene organization of *CaDEF* and *CaDEFL* genes. The boxes represent exons, dotted-lines represent introns, and the double-lines represent UTR

variants, i.e., products of alternative splicing. While group CaDEF2 had eight members, of whom CaDEF2.5a and CaDEF2.5b were identified as transcript variants, CaDEFL group constituted of six members (CaDEFL1, CaDEFL2, CaDEFL3 CaDEFL4, CaDEFL5, and CaDEFL6). Five phylogenetically distant CaDEFs that did not fit in any of these groups were classified as CaDEF3, CaDEF4, CaDEF5, CaDEF6, and CaDEF7.

The phylogenetic analysis of CaDEFs with homologs from *A. thaliana* and *M. truncatula* revealed a close evolutionary relationship between CaDEF1 and MtDEF2.1, while different members of group CaDEF2 were closer to AtPDF2.5, AtPDF2.6, and MtDEF4 proteins (Supplementary Fig. 1a). While the evolutionary relationship of CaDEFLs with other defensin-like proteins was studied by aligning 300 DEFL proteins from *A. thaliana* and 49 DEFL proteins from *M. truncatula*, constructing the phylogenetic tree was a challenge since the distance between several pairs of alignment could not be calculated with the MEGA7 software. To overcome this, a small sub-tree was generated based on similarities between CaDEFLs, AtDEFLs, and MtDEFLs that revealed clustering of all six CaDEFLs in a distinct clade (Supplementary Fig. 1b). The bootstrap values indicated strong confidence in phylogenetic relationship of the DEFL families and established that CaDEFLs had comparable homologs in the model plant species.

Sequence Analyses and Characterization

All 22 chickpea DEF and DEFL protein sequences exhibited 8–15 characteristic Cys residues with the signature CSαβ motif revealing highly conserved organizational patterns between the 3rd and 4th Cys residues. Multiple alignment of protein sequences showed conservation of Cys and Gly residues (Fig. 2). The group-wise multiple sequence alignments indicated very high degree of conservation. CaDEF1.1a, CaDEF1.1b, and CaDEF1.1c were found to be products of transcript variants with 98% sequence identity among these. The group 1 mature protein sequences showed signature CSαβ motif and conserved 8 Cys residues with a $C_1X_{10}C_2X_5C_3X_3C_4X_{10}C_5X_5C_6X_1C_7X_3C_8$ pattern. Similarly, members of CaDEF2 shared varying identities with each other (48–97%); CaDEF2.5a and CaDEF2.5b being products of transcript variants. Group 2 mature protein sequences were again highly conserved with a CSαβ motif, ɣ-thionin core and $C_1X_{10}C_2X_5C_3X_3C_4X_9C_5X_6C_6X_1C_7X_3C_8$ Cys residue pattern which differed in pattern from group 1 with respect to number of amino acids between C_4 and C_6. Interestingly, group DEFL included 127 to 130 amino acid long peptides that showed 37–81% identity within the group. These contained 15 conserved Cys residues with a pattern $C_1X_6C_2X_{3\text{-}4}C_3X_3C_4X_{11-13}C_5X_5C_6X_1C_7X_{8-13}C_8X_6C_9X_3C_{10}X_4C_{11}X_3C_{12}X_1C_{13}X_{10}C_{14}X_1C_{15}$ (Fig. 2).

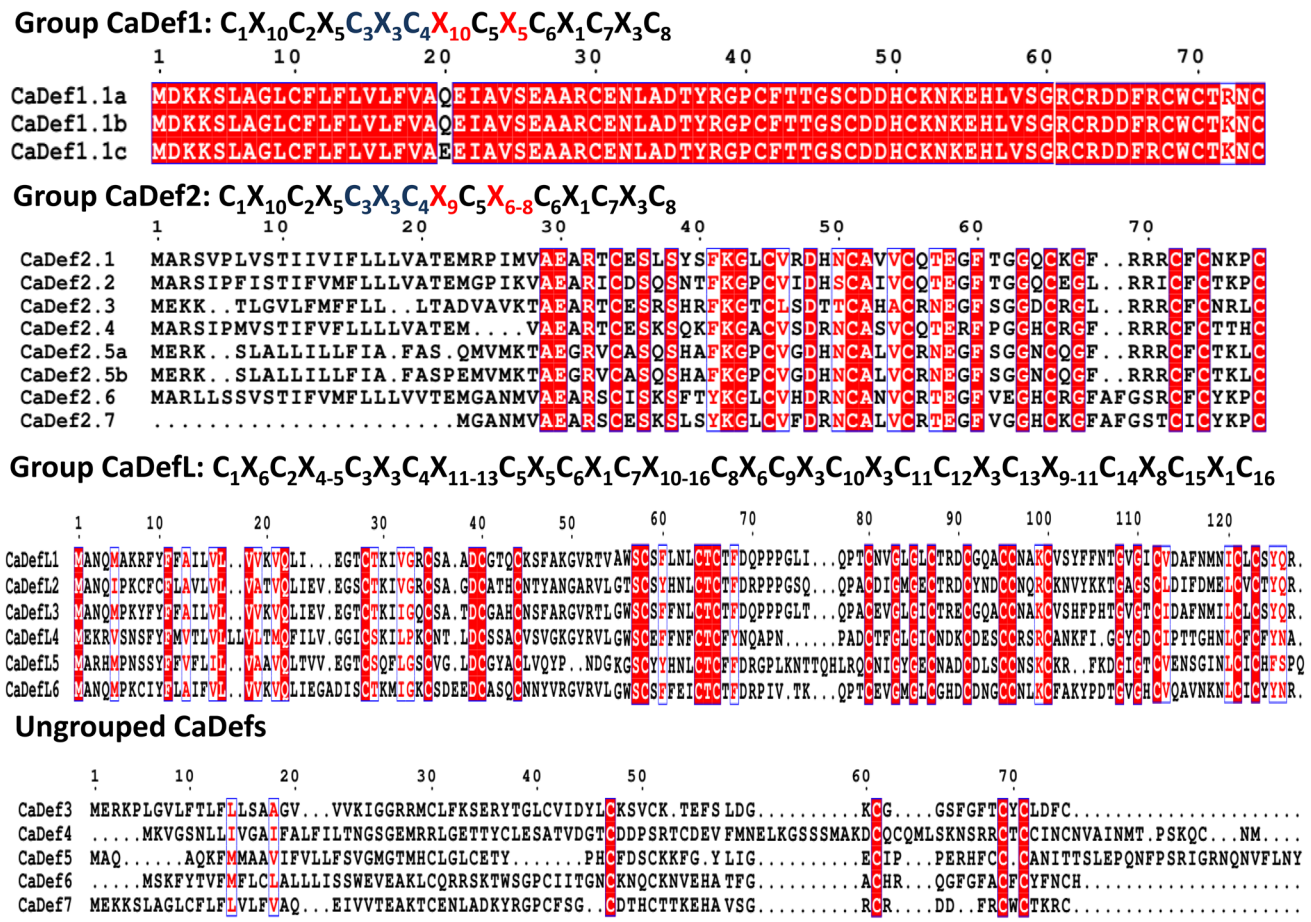

Fig. 2 Sequence analysis and genomic organization of CaDEF and CaDEFLs. Multiple sequence alignment of CaDEF and CaDEFL amino acid sequences generated by using ESPript showing the distinctive CSαβ domain characterized by CX_3C sequences

The conserved motifs in CaDEF and CaDEFL proteins were studied using the MEME suite to obtain insights into the diversity of composition (Supplementary Fig. 2). A total of five conserved motifs were identified and designated as motif 1–5. Motif 1 belonged to the gamma-thionin family (pfam00304), a close relative of the defensin family that was distributed in all members of group 1 and group 2 along with CaDEF3 and CaDEF6 (Supplementary Fig. 3). Motif 2, representing the signal peptide was found to be present near the N-terminal of almost all CaDEF and CaDEFL proteins with exceptions being CaDEF2.7 and CaDEF6 that lacked the signal peptide. Although the motif 4 was found to be present in all defensins of group1 and 2, CaDEF3, CaDEF6, and CaDEF7, its function remained ambiguous. The motifs 3 and 5 were found to be distributed in all CaDEFL proteins (Supplementary Fig. 3). The putative function of these motifs could not be identified, and their functional characterization may shed some light upon the role of these motifs. Intriguingly, the phosphatidic acid (PA) binding RGFRRR motif, that has been reported to facilitate internalization of defensin by interacting with pathogen cell walls, was found only in CaDEF2.4. Other members of group 2 showed variations in the RGFRRR motif which could be depicted by XG(F/L) RRX.

The identified proteins were characterized in silico with respect to their molecular weight, isoelectric point, chromosomal location, and subcellular localization. The predicted molecular weights ranged from 6.2 to 14.4 kDa, while their pI varied from 6.05 to 9.14 (Table 1). While, CaDEF2.4, CaDEF2.7, CaDEF3, CaDEFL4, and CaDEFL5 were predicted to localize in the chloroplast, rest of the CaDEF and CaDEFL peptides were predicted for extracellular targeting.

Gene Structure Organization and In Silico Promoter Analysis

The structural diversity of *CaDEF* genes was identified by aligning the genomic and coding region sequences using GSDS (Fig. 1). While majority of *CaDEFs* contained two exons interrupted by an intron, *CaDEF2.7* and *CaDEF7* contained a single exon. The length of exon 1 ranged from 57 to 66 nucleotides in all *CaDEF* genes, thereby indicating

Table 1 General properties of CaDEF proteins

S.No	Gene name	Protein ID	CDS (bp)	Protein (AA)	Molecular weight (Da)	pI	Chromosome		Intron	Upstream region (bp)	TMH	Subcellular localization
							Location	Position				
1	CaDEF1.1a	XP_004487695.1	225	74	8370.6	6.68	1	16,939,546..16940089	1	76	1	s
2	CaDEF1.1b	ABC59238.1	225	74	8342.5	6.68	1	16,939,546..16940089	1	746	1	s
3	CaDEF1.1c	ABC02867.2	225	74	8343.5	6.05	1	16,939,546..16940089	1	746	0	s
4	CaDEF2.1	XP_004509603.1	237	78	8664.3	8.93	7	17,483,097..17483701	1	42	1	s
5	CaDEF2.2	XP_004509604.1	237	78	8493	7.52	7	17,488,511..17489106	1	37	1	s
6	CaDEF2.3	XP_012572202.1	225	74	8298.6	9.12	6	6,479,979..6481058	1	50	0	s
7	CaDEF2.4	XP_004509606.1	225	74	8281.7	9.14	7	17,530,925..17531957	1	111	1	s
8	CaDEF2.5a	XP_004503015.1	225	74	8119.6	9.12	5	47,961,484..47962145	1	102	1	s
9	CaDEF2.5b	XP_004503014.1	228	75	8217.7	8.93	5	47,961,484..47962145	1	102	0	s
10	CaDEF2.6	XP_004509602.1	243	80	8872.4	8.72	7	17,437,620..17438223	1	–	1	s
11	CaDEF2.7	XP_012573798.1	177	58	6208.2	8.48	7	17,346,485..17346661	0	–	0	NA
12	CaDEF3	XP_012572203.1	228	75	8296.9	8.68	6	6,505,704..6508173	1	21	1	s
13	CaDEF4	XP_012568839.1	309	102	11,107.8	6.15	3	7,771,463..7771985	1	57	1	s
14	CaDEF5	XP_012575680.1	246	87	9835.6	7.56	UGS	41,931..42407	1	10	1	s
15	CaDEF6	XP_004510254.1	231	76	8727.2	8.82	7	30,238,811..30239433	1	27	1	s
16	CaDEF7	AAO38756.1	219	72	8171.5	8.1	UGS	–	0	–	1	s
17	CaDEFL1	XP_012573931.1	384	127	13,787.2	8.61	1	19,688,339..19688933	1	13	0	s
18	CaDEFL2	XP_012573922.1	390	129	14,007.1	6.65	1	19,715,004..19715819	1	19	0	s
19	CaDEFL3	XP_012573929.1	390	129	14,026.4	7.39	1	19,671,770..19672840	1	15	0	s
20	CaDEFL4	XP_004512205.1	384	127	14,039.4	7.85	8	3,189,597..3190217	1	95	1	s
21	CaDEFL5	XP_004513498.1	390	129	14,197.3	6.83	UGS	662,594..663621	1	–	1	s
22	CaDEFL6	XP_012573935.1	393	130	14,424.8	6.66	1	19,678,421..19679635	1	19	0	s

that these were highly conserved. The intron and exon 2 lengths varied. Three types of introns were identified: phase-0 (intron exactly between two codons), phase-1 (intron between first and second base of codon) and phase-2 (intron between second and third base of codon), revealing five genes with phase-0 introns and nine genes with phase-1 and 6 with phase-2 introns.

Many *cis*-acting elements specific to wound stress, abiotic stress and tissue-specific expression were identified in *CaDEF* and *CaDEFL* promoters (Table 2). These included the wound-responsive element (WUN-motif) responsible for response to pathogen and elicitors (TC-rich repeats, Box W1 and ELI-box3), salicylic acid and methyl jasmonate-responsive elements (TCA, TGACG and CGTCA motifs). Other relevant cis-regulatory elements identified in most *CaDEF* promoters included ABA-responsive element (ABRE), heat shock element (HSE), low temperature-responsive (LTR), MYB binding site involved in drought-inducibility (MBS), gibberellin response element (GARE), and auxin-responsive element (TGA) (Table 2).

Synteny Analysis and Chromosomal Location

Synteny analysis of *CaDEF* genes revealed that *CaDEF1* genes on chromosome 1 shared synteny with *Defensin* genes located on chromosome 1 and chromosome 2 of *A. thaliana* and *M. truncatula*, respectively (Fig. 3). Similarly, *CaDEFL* genes clustered on chromosome 1 shared synteny with genes localized on chromosome 2 in *M. truncatula* and chromosome 4 of *A. thaliana*. Furthermore, *CaDEF2* genes clustered on chromosome 7 showed syntenic relationships with *AtDEF* genes on chromosome 2 but did not indicate any relationship with *M. truncatula*.

Moreover, chromosomal location analyses of *CaDEF* and *CaDEFL* genes revealed distribution of 13 *CaDEF* and *CaDEFL* genes on 6 out of 8 chromosomes of chickpea, while the remaining 2 genes were mapped to unplaced genomic scaffolds (Fig. 3). While several of these genes were localized on chromosome numbers 3, 5, 6, and 8, *CaDEFL* genes clustered together in a 43 kb region on chromosome 1, between the co-ordinates 19671770 and 19715819, closer to *CaDEF1* group cluster in the vicinity at 16939546–16940089. Another high-density cluster of five genes belonging to group 2, viz., *CaDEF2.1, CaDEF2.2, CaDEF2.4, CaDEF2.6a, and CaDEF2.7*, that lied in a 185 kb region on chromosome 7 between the co-ordinates 17346485 and 17531957. These gene clusters indicated the occurrence of strong local gene duplication.

The *CaDEFL* cluster on chromosome 1 and *CaDEF* cluster on chromosome 7 were studied in detail to identify their significance in stress response mechanisms and for determining the flanking genes (Supplementary Fig. 4). The *CaDEFL* cluster upstream was flanked by genes encoding proteins such as nifU-like protein 3 (XP_004487950.1), TOX-4-like (XP_012573704.1), glucose-6-phosphate 1-epimerase (XP_004487963.1), villin-4-like (villin-4-like), METAXIN-like (XP_004487965.1) and pentatricopeptide repeat-containing protein (PPR) (XP_004487967.1). The downstream region comprised of PPR (XP_012573978.1), oxysterol-binding proteins (XP_004487976.1, XP_004487979.1), subtilisin-like protease (XP_012573973.1), DNA-directed RNA polymerase I subunit (XP_012573983.1) and serine/threonine-protein phosphatase (XP_004488005.1), with a few uncharacterized genes distributed in between (Supplementary Fig. 4, Table S2).

Similarly, the upstream region of the *CaDEF* cluster on chromosome 7 exhibited genes encoding proteins such as isoprenylcysteine alpha-carbonyl methylesterase (XP_004509595.1), six PPR transcript variants (XP_004509598.1, XP_004509599.1, XP_012573810.1, XP_012573811.1, XP_012573812.1, and XP_012573813.1), histone-lysine N-methyltransferase (XP_004509600.1), aldo–keto reductase (XP_004509601.1). Whereas, the downstream region contained genes coding for proteins such as GDSL esterase/lipase (XP_004509605.1), UBX domain-containing protein (XP_004509607.1), transcription factor GTE1 (XP_004509610.1) and actin-related protein 2/3 complex (XP_004509613.1; Supplementary Fig. 4, Table S2).

Expression of *CaDEF* Genes During Pathogen Attack

The expression profiles of the identified chickpea *defensin* genes were investigated in root tissues challenged with *F. oxysporum* and *R. bataticola*, causal agents of *Fusarium* wilt and dry root rot diseases, respectively. qRT-PCR analyses of infected tissues were carried out to investigate differential expression of *CaDEF* and *CaDEFL* genes in resistance to *F. oxysporum* infection using resistant (WR 315) and susceptible (JG 62) chickpea varieties at different stages of disease progression (Table S1). Results suggested that at early stages i.e., 24 h post-inoculation (hpi), *CaDEF3, CaDEF4, and CaDEF7* showed an increased expression in the susceptible genotype JG 62 alone, while other genes such as *CaDEF2.4* and *CaDEF2.5b,* showed enhanced expression in both genotypes. Although no major induction occurred in JG 62 after 48 h, the resistant genotype WR 315 showed up-regulation of *CaDEF1.1a*, *CaDEF1.1b*, *CaDEF1.1c, and CaDEF2.4*. Interestingly, *CaDEF2.5b* was down regulated in both the tested genotypes at 48 hpi.

Intriguingly, the resistant genotype WR 315 showed a significant increase in the expression of all *CaDEFs* from 48 h onwards (except *CaDEF2.5b, CaDEF2.6*, and *CaDEF7*), reaching maximum expression at 120 hpi, which thereafter decreased by 288 hpi. No disease symptoms were observed in this genotype until end of the experiment. In contrast, JG 62 did not exhibit significant changes in the expression

Table 2 Cis-regulatory elements from the promotor region of CaDEF genes

S.No	Gene	ABRE motif	MBS	TC-rich repeats	WUN-motif	ERE	TCA-element	TGACG-motif	CGTCA-motif	Box-W1	ELI-box3
1	CaDEF1.1a	9 (+104) 6 (+104)	6 (+114) 6 (+1370) 6 (−1349)	9 (+430) 9 (+1271) 9 (−788)	9 (−1209)						
2	CaDEF1.1b			9 (+193)		8 (+117)	9 (+8)				
3	CaDEF1.1c			9 (+193)		8 (+117)	9 (+8)				
4	CaDEF2.1		6 (−348)	9 (−1240)							
5	CaDEF2.2	6 (+262)				8 (−1241)		5 (+750)	5 (−750)		9 (−148) 9 (+764)
6	CaDEF2.3	9 (−1275)	6 (+998)	9 (−66)		8 (+352) 8 (+379)	9 (−797)	5 (−1373)	5 (+1373)	6 (+567)	
7	CaDEF2.4			9 (+139)			9 (+797)	5 (+453)	5 (−453)		
8	CaDEF2.5a			9 (+1374)		8 (+246)					
9	CaDEF2.5b			9 (+1374)		8 (−246)					
10	CaDEF2.6					8 (+1443)				6 (−335) 6 (+415)	
11	CaDEF2.7		6 (+1059) 6 (−1155)				9 (−513) 9 (−578)	5 (−205)	5 (+205)		
12	CaDEF3		6 (+153)	9 (+667) 9 (−1233) 9 (−880)						6 (+519)	
13	CaDEF4					8 (−987).8 (−1023)	9 (+1109) 9 (−1193)				
14	CaDEF5	6 (+199)	6 (+1481)	9 (+943)			9 (−886)				
15	CaDEF6			9 (+351)		8 (+193)	9 (+145)				
16	CaDEFL1	6 (−1379)	6 (+786)	9 (−139)							
17	CaDEFL2		6 (+962) 6 (+1102)	9 (−354)	9 (−558)			5 (−17) 5 (−26)	5 (+17) 5 (+26)	6 (−963) 6 (+1407) 6 (−1103)	
18	CaDEFL3		6 (−320)	9 (−858)						6 (−65) 6 (−813)	9 (+56)
19	CaDEFL4		6 (+414)			8 (−206)	9 (+102)				
20	CaDEFL5	6 (−1402) 7 (+1403)		9 (−585)			9 (+229) 9 (+508) 9 (−416) 9 (+368) 9 (+425)	5 (−166) 5 (+466)	5 (+166) 5 (−466)	6 (−1313)	
21	CaDEFL6	6 (−563)	6 (−375) 6 (−1081)	9 (−579) 9 (+685)		8 (−502) 8 (−954)				6 (+1344) 6 (+1410)	

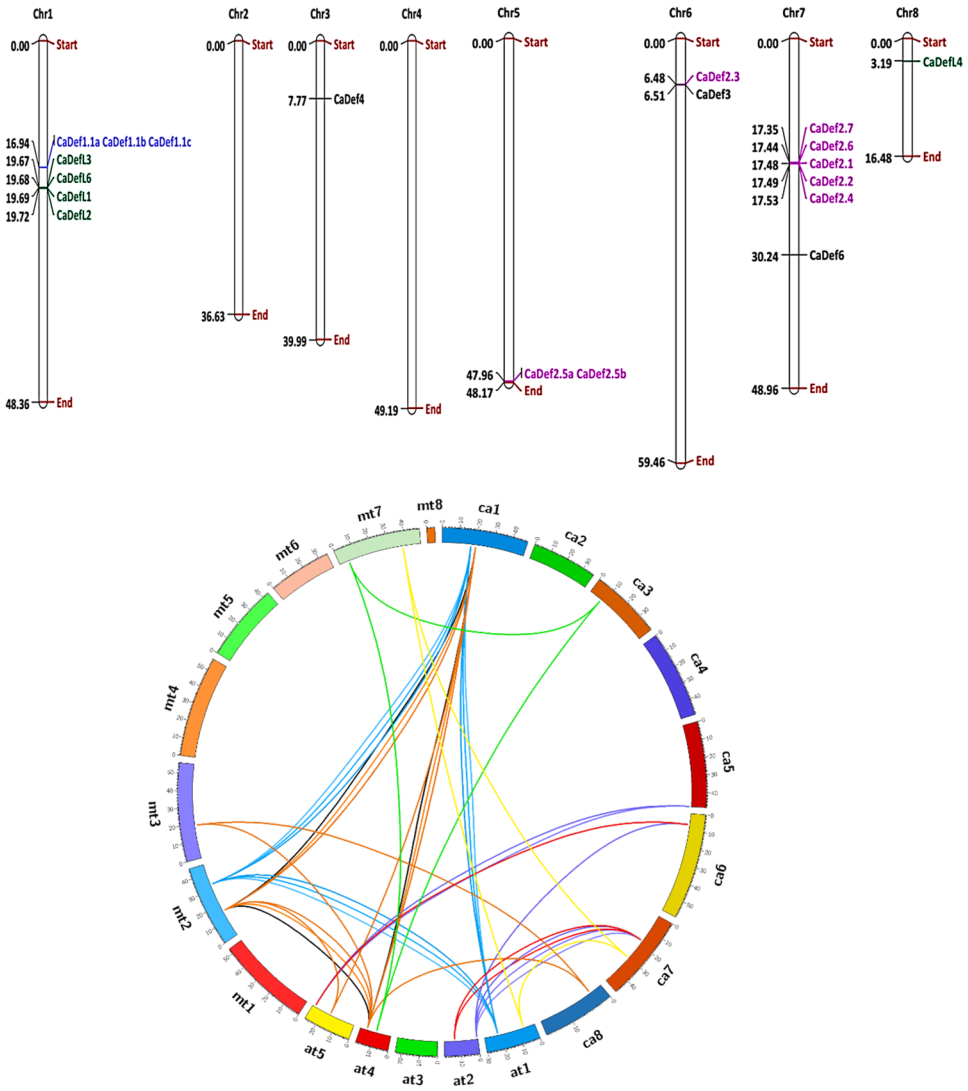

Fig. 3 Chromosomal location and homologous gene relationships among *DEF and DEFL* genes from *Arabidopsis thaliana*, *Medicago truncatula and Cicer arietinum*) The chromosomal location information of *Defensin and Defensin-like* genes was obtained from NCBI and mapped to chickpea chromosomes. The map depicting chromosomal locations was generated using MapChart 2.0. The color of each gene depicts corresponding group. Homologs of each *CaDEF* and *CaDEFL* gene were determined manually by conducting BLAST searches against *A. thaliana* and *M. truncatula* whole genome sequences. The synteny map was generated using Circos v0.69 (Color figure online)

of any of these *CaDEF* genes throughout the experimental duration and showed medium to heavy wilt symptoms 120 hpi onwards (Fig. 5a).

Using the co-relation regression line between the logarithm of tenfold serial diluted known Foc DNA concentrations and qRT-PCR threshold cycles (Cts) of each DNA, the Foc colonization was measured within the root tissue to determine the wilt disease establishment in chickpea. There was no Foc colonization was detected in the root tissue up to 4 dpi. After 7 dpi a detectable amount of Foc DNA was measured in both cv. JG 62 and WR 315. Thereafter, the amount of the Foc DNA increased significantly in both cultivars at 12 dpi and reached up to 0.821 ng and 0.155 ng per 10 ng of root tissue DNA of JG 62 and WR 315, respectively. The growth of Foc was comparatively slower in WR 315 than JG 62. At 15 dpi, the relative quantity of Foc DNA was started to decline reasonably in both the cultivars, as seedlings of WR 315 became inured with disease and started to acclimatize at late time point, whereas in JG 62, the seedling were severely drooped or died completely, consequently the reduction of Foc DNA in the roots of JG 62 at the same time point (Fig. 4). In an analogous experiment, three chickpea genotypes, showing differential resistance to dry root rot disease caused by *R. bataticola,* were compared with respect to the gene expression profiles of selected *CaDEF*s (showing higher expression in the resistant genotype in response to *Fusarium* wilt). Based on the greenhouse assays (Table 3), ICC 05530 was considered to possess moderate tolerance to the pathogen, whereas wild chickpea progenitor, *C. reticulatum* (ICC 17160) and BG 212 were categorized as resistant and susceptible checks, respectively. The induction of chickpea defensins upon pathogen infection was assayed in inoculated 10–12 day old chickpea seedlings. Similar to the *Fusarium* assays, *CaDEF2.5a* and *CaDEF2.5b* exhibited early up-regulation after 72 hpi in all genotypes. However, the extent of expression varied among the tested genotypes,

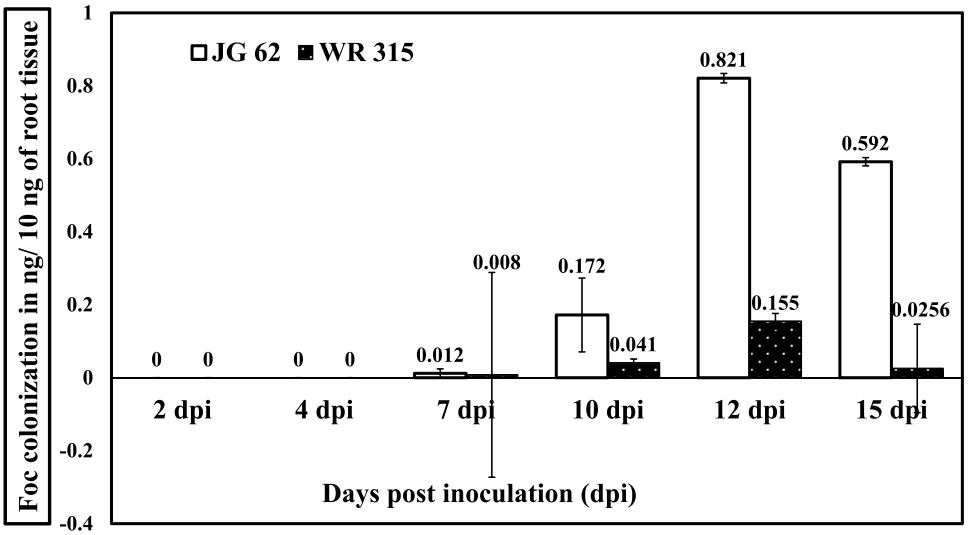

Fig. 4 Evaluation of disease progression of Fusarium wilt. A graph showing Foc colonization (ng/10 ng of root tissue) against the days 2, 4, 7, 10, 12, and 15 post-inoculation (dpi)

Table 3 Evaluation of disease progression and relative resistance of Fusarium wilt and dry root rot in susceptible and resistant chickpea genotypes under greenhouse conditions

Genotype	Disease incidence (%) of *Fusarium* wilt in hours post-inoculation (hpi)[a]							
	24 hpi	48 hpi	72 hpi	120 hpi	168 hpi	240 hpi	288 hpi	360 hpi
JG 62	0	0	0	0	13.33	53.33	80	100
WR 315	0	0	0	0	0	0	0	0

	Disease severity of Dry Root Rot (1–9 scale) in hours post-inoculation (hpi) #				
	24 hpi	48 hpi	72 hpi	120 hpi	168 hpi
BG 212	0	0	2.3	6	8.6
ICCV 05,530	0	0	0	2.3	4.3
C. reticulatum (ICC 17,160)	0	0	0	0.6	3.3

[a]Results are mean of data from three replications, each containing > 5 individual plants

with ICC 05530 having a significantly higher expression than the other two genotypes (BG 212 and *C. reticulatum*; Fig. 5b). At day 5, the expression of *CaDEF2.5a* and *CaDEF2.5b* subsided in BG 212 and ICC 05530 whereas, *C. reticulatum* (ICC 17160) revealed a steady expression of these genes. The expression of *CaDEF2.4, CaDEFf2.5a, CaDEF2.5b, CaDEFL2, CaDEF4,* and *CaDEF5* consistently increased in *C. reticulatum* (ICC 17160) throughout the experiment (Fig. 5b). These results were statistically significant with %CV < 15%.

Besides, studying the expression profiles of various *CaDEF* genes in response to *F. oxysporum* f. sp. *ciceris* infection, we also studied the fungal response to defensin activity by analyzing the differential expression of four of its genes involved in fungal cell wall synthesis i.e., *Beta-1,3-glucanosyltransferase* (*GAS1*), *ethanolaminephosphotransferase (LAS21), 1,3-beta-glucan synthase (FKS1)* and *Switching-deficient* transcription co-factor (*SWI6)* (Accession No. AY884608.1, JH651387.1, EWZ01533.1 and EWZ35589.1, respectively). *F. oxysporum* infecting the resistant genotype WR 315 did not show significant changes in transcript abundance of GAS*1, LAS21, FKS1*, and *SWI6* until 120 hpi, with *LAS21* showing slight transcript changes at 288 hpi. In contrast, *F. oxysporum* infecting the susceptible genotype JG 62 had the transcript levels that were similar to the resistant genotype until 48 hpi, and increased thereafter until 288 hpi, when heavy wilt symptoms were observed in these test genotypes (Fig. 5c). Several defensin and defensin-like genes such as *CaDEF1.1b, CaDEF2.5a, CaDEF3, CaD*EF5, and *CaDEFL2,* that showed enhanced expression in response to both *Fusarium* wilt and dry root rot pathogens, were chosen for molecular docking studies.

Protein Structure and Interactions with Fungal Plasma Membrane Components

The 3D structures of selected CaDEF proteins were predicted using the homology modeling. The MODWEB

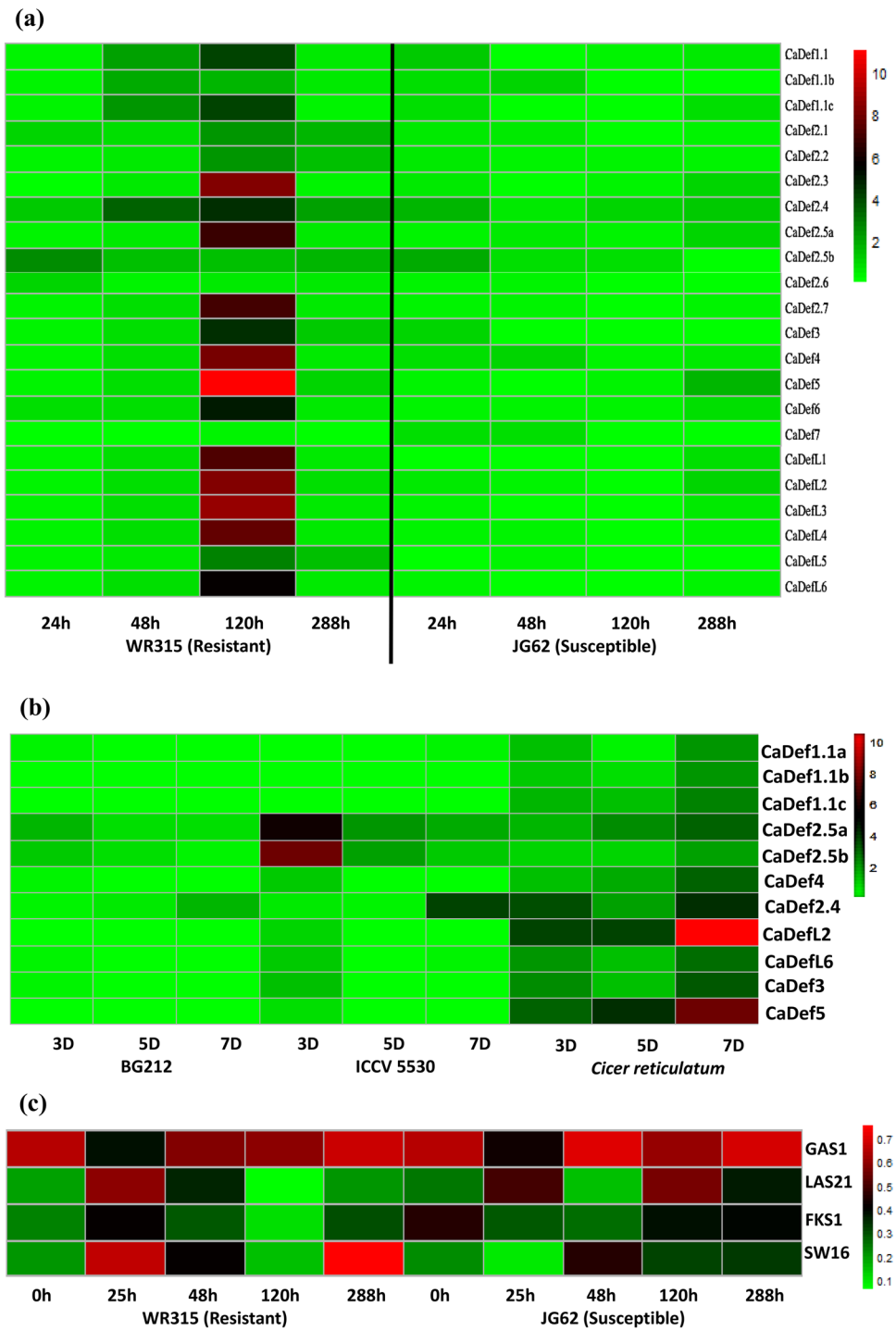

Fig. 5 Expression of *CaDEF* and *CaDEFL* genes in cultivated and wild chickpea species following pathogen invasion. **a** Relative expression levels of chickpea *Def* gene family upon *F. oxysporum* f. sp. *Ciceris* infection in the resistant genotype WR 315 and the susceptible genotype JG 62 at different time points. **b** Expression of selected *CaDEF* and *CaDEFL* genes in the root tissue of BG 212, ICCV 05,530 and the wild species *C. reticulatum* in response to *R. bataticola* infection. **c** Transcript levels of *F. oxysporum* f. sp. *Ciceris* genes, in infected root samples over a period of 288 hpi

server could model structures for CaDEF1.1b, CaDEF2.5a, CaDEF3, CaDEF5, and CaDEFL2, based on availability of Protein Data Bank (PDB) templates having high percentage sequence identity and query coverage. The proteins were superimposed upon their respective templates with 100% structure overlap (SO) with root mean square deviation (RMSD) ranging from 0.17 to 1.22 (Table S3). The interaction of CaDEFs with components of fungal plasma membrane, particularly with phosphatidylserine (PS) and glucosylceramide (GluCer) were determined by identifying putative binding sites. To identify the putative PS binding site, each of the 5 CaDEF models were superimposed on PDB ID—4CQK, chain-A, (representing crystal structure of *Nicotiana alta* defensin 1 (NaD1) bound to phosphoinositide, that shares structure similarity with PS) (Table S4). Residues whose C-alpha and C-beta atoms superimposed

with corresponding atoms of residues from 4CQK-A within a distance of 5 Å from phosphoinositide (residues- 4, 33, 34, 35, 36, 37, 38, 39, 40, 42—from 4CQK-A) were considered to be putative interacting agents (Table S5). Molecular docking analyses of the 5 CaDEF models with PS revealed the following interacting residues: CaDEF1.1b—29-R, 30-C, 31-E, 61-R, 63-R, 64-D, 65-D; CaDEF2.5 -31-A, 60-N, 61-C, 62-Q, 69-F, 71-T; CaDEF3: 30-C, 32-F, 36-R, 37-Y, 38-T, 40-L, 64-S, 65-F, 66-G, 67-F, 68-T; CaDEF5—52-I, 53-P, 54-P, 55-E, 57-H, 60-C; CaDEFL2—27-E, 28-G, 29-S, 59-S, 60-C, 61-S, 32-Y, 63-H, 65-L, 67-T (Fig. 6). Incidentally, our attempts to identify putative residues interacting with GluCer were limited due to the unavailability of defensin-GluCer crystal structure. However, PDB ID-2KSK, that depicted NMR based interaction studies between GluCer and sugarcane defensin, was used as a template for superimposition of selected 5 CaDEF models, that determined the putative residues that bind to GluCer (residues—31, 32, 34, 35, 36, 37) (Table S6).

Discussion

A homology and HMM-based approach were used to identify defensins that might play a crucial role in host-plant resistance in chickpea. Our study revealed 16 defensins (CaDEF) and 6 defensin-like (CaDEFL) proteins in chickpea. Although, the possibility of existence of additional defensin genes with distant homologies is highly improbable, it cannot be ruled out. Similar studies have reported 15–50 defensins from other plant genomes (Silverstein et al. 2005; Thomma et al. 2002). Based on the phylogenetic analysis, *CaDEF* genes were classified further into groups, each exhibiting various conserved domains that may bestow variable functional characteristics such as defensin hallmark ɣ-core motif that confers antibacterial and antifungal properties (Bohlmann and Broekaert 1994). Major determinants of the antifungal activity and morphogenicity of defensins have been reported to reside in their γ-core motifs; although, minor determinants outside the γ-core motifs also contribute to their antifungal activity (Sagaram et al. 2013). The evolutionary relationship of CaDEFs and CaDEFLs with their counterparts in model plant species indicated a closer relationship with *MtDEF* genes as compared to *AtDEF*. Most members belonging to different sub-families were clustered together in clades suggesting that multiple members belonging to a CaDEF sub-family must have arisen due to gene expansion through tandem duplications resulting from unequal crossing over, leading to small changes in the gene sequences (Cannon et al. 2004) resulting in multifunctional roles (Thomma et al. 2002).

The structure of *CaDEF* gene shows presence of a single intron after 54–66 nucleotides long exon 1 that seems to be critical for intron-mediated enhancement (IME), frequently

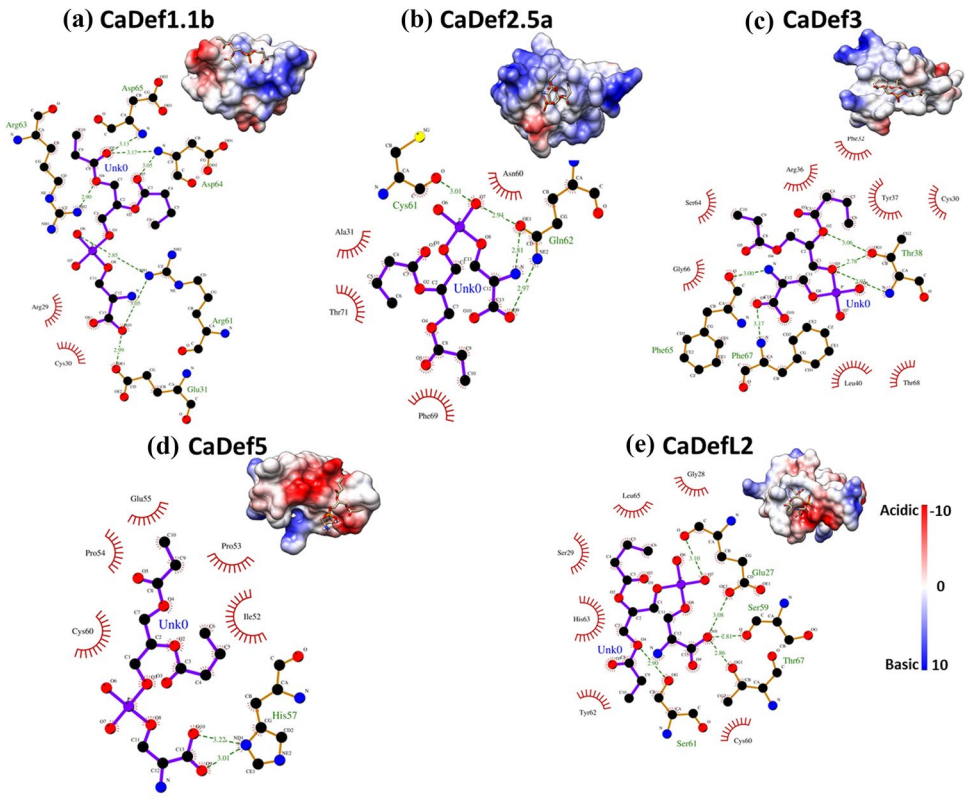

Fig. 6 Molecular docking revealing interactions of CaDEF and CaDEFL proteins with phosphatidylserine (PS), a fungal cell wall component. **a** CaDEF1.1b, **b** CaDEF2.5a, **c** CaDEF3, **d** CaDEF5, and **e** CaDEFL2. The electrostatic interactions are depicted (inset) and corresponding LigPlots show residues involved in electrostatic and hydrophobic interactions

observed in plants (Rose 2008). Moreover, its significance in the *CaDEFs* predominantly expressing under pathogen attack explains the invasion-induced gene expression possibly due to an intron-mediated boost in the pre-mRNA synthesis by increasing transcription initiation and RNA polymerase II processing (Furger et al. 2002; Kwek et al. 2002; Proudfoot et al. 2002). Further, the analysis of promoter regions of *CaDEF* genes was also carried out for presence of cis-regulatory elements that provided indirect functional evidences for their role in response to stress conditions (Lynch and Conery 2000). Interestingly, methyl jasmonate (TGACG and CGTCA motifs) and salicylic acid (TCA motif) responsive elements identified in the promoter regions of some *CaDEF* genes signify the key roles of *CaDEF* genes in resistance-associated mechanisms, some of which may be modulated by different stress-induced hormones.

CaDEF and *CaDEFL* genes were found to be distributed on all chickpea chromosomes except chromosomes 2 and 4, where most showed a homology-based pairwise synteny with defensins from *A. thaliana* and *M. truncatula* as observed in several previous reports (Jain et al. 2013; Varshney et al. 2013; Benko-Iseppon et al. 2003). The chromosomal localization of *CaDEF* genes indicated their existence in clusters. Clustering of genes involved in defense response is a common feature in plant genomes (Yang and Wang 2016) that has been reported earlier in various organisms such as chicken, humans, and *Arabidopsis* (Hollox et al. 2003; Xiao et al. 2004; Takeuchi et al. 2010). The two clusters present on chromosomes 1 and 7 in chickpea contain genes that are closely related to each other providing a reservoir of genetic variation, suggesting that rapid molecular evolution by local duplication and positive selection could have formed such gene clusters (Silverstein et al. 2005; Maxwell et al. 2003; Semple et al. 2003; Graham et al. 2004). Different expression patterns of genes belonging to the same family imply possible functional redundancy or diversification (Innan and Kondrashov 2010). Additionally, tandem duplications of *CaDEF* groups may result in increased transcript and protein accumulation or functional diversification. Indeed, the elevated expression of *CaDEFL* genes may be due to a high copy number.

Interestingly, some of the genes flanking the two identified chickpea defensin clusters such as PPR, oxysterol-binding protein, subtilisin-like protease and serine/threonine-protein phosphatase have been known to play diverse roles in plant defense mechanisms, mostly for abiotic and biotic stress responsiveness. Overall, this highlighted our hypothesis that the two clusters of physically adjacent genes co-express and contributes to stress response mechanisms (Williams and Bowles 2004; Mezey et al. 2008). The proximity, common cis-regulatory elements, promoter and enhancer regions, and chromatin-level regulation could possibly explain the correlation of gene clusters with expression (Oliver et al. 2002). Future studies on transcriptional activities and segregation of these regions in various disease resistant and stress adapted chickpea genotypes could provide leads on a 'stress responsive hotspot', which potentially can be introgressed to develop varieties with improved resistance traits.

The expression profiles of *CaDEF* genes using qRT-PCR in response to two major fungal pathogens, (*F. oxysporum* f. sp. *ciceris,* a hemi-biotrophic fungi and *Rhizoctonia bataticola, a* necrotroph that colonizes chickpea roots) revealed differential expression of individual defensins from resistant and susceptible chickpea genotypes warranting further studies to analyze their overexpression in the host-plant resistance. Overexpression of defensins such as wasabi defensin (WT1) in rice, potato, and orchids have shown increased resistance to *Magnaporthe grisea, Erwinia carotovora, F. oxysporum* and *Botrytis cinerea* (Lay and Anderson 2005; Kong et al. 2014), and *RsAFP-1* defensin gene from mustard in transgenic tobacco and peanut plants showed enhanced resistance to fungal pathogens *F. moniliforme*, *P. parasitica, P. nicotianae* and *C. arachidicola* (Anuradha et al. 2008). Similarly, while expressing Dahlia defensin (Dm-AMP1) in rice inhibited the pathogen, *M. oryzae* and *Rhizoctonia solani* (Jha et al. 2009), extracellular, vacuolar and ER localization of *MtDEF4.2*, a defensin from *M. truncatula* in *A. thaliana* resulted in enhanced resistance to *Fusarium* wilt (Kaur et al. 2011). Our experimental studies confirmed that six defensin genes, viz. *CaDEF1.1b, CaDEF2.4, CaDEF2.5a, CaDEF3, CaDEF5*, and one *CaDEFL2* gene that showed significant up-regulation during in genotypes resistant to both *Fusarium* and *R. bataticola* infection, and potentially have a role in their innate immune response. Considering that some of these *CaDEFs* might be a result of gene duplication events, their diverse expression profiles might be attributed to sub-functionalization, (splitting of multiple functions of the original gene between the two duplicated genes) or neo-functionalization (developing a novel function).

We also studied the expression of *F. oxysporum* genes viz. *GAS1, LAS21, FKS1*, and *SWI6* that are involved in fungal cell wall synthesis and maintenance during infection and disease progression (García et al. 2015). Our results revealed that infecting the resistant genotype WR 315 with *Fusarium* significantly lowered the expression of *GAS1, FKS1, LAS21,* and *SWI6* genes compared to the susceptible genotype JG 62 throughout the duration of the experiment (until 288 hpi). While *GAS1* localizes to cell surface by anchoring to glycosylphosphatidylinositol and is required for cell wall assembly, morphogenesis, and cell wall integrity, *FKS1* represents catalytic subunit of 1,3-beta-D-glucan synthase that is responsible for cell wall synthesis and maintenance. *SWI6*, a transcription co-factor regulates transcription at the G1/S transition, whereas *LAS21* codes for an integral plasma membrane protein. These genes are transcriptionally induced

under cell wall stress conditions for aiding recovery from stress and boosting fungal growth (García et al. 2015). Interestingly, transcript abundance of these genes shows a strong negative correlation with the expression of *CaDEF* genes in resistant as well as susceptible genotypes, indicative of their putative role in defense against fungal pathogen.

The activity of AMPs depends upon the composition and physicochemical properties of cell surface layer (Lacerda et al.2014; Matsuzaki 1999). To further study the interaction of defensins with the cell membrane residues of invading pathogens, we looked at the electrostatic interaction of these with negatively charged lipid bilayer components characteristic of any bacterial and fungal pathogens. Since defensins do not present a distinct pattern of positively charged or hydrophobic amino acids, their secondary structure and disulphide bridges-mediated folding contribute to their antimicrobial activity (Matsuzaki 2009). Recently, the RGFRRR motif from MtDef4 was reported to play a role in protein internalization and membrane disruption (Sagaram et al. 2013). However, this motif did not exist in CaDEFs and CaDEFLs, suggesting that multiple mechanisms govern the activity of defensins, in line with many other reports (Vriens et al. 2014; Yang et al. 2001; Rotem and Mor 2009). Moreover, our molecular docking data with the 5 selected chickpea defensins revealed differences in charge and hydropathicity of interacting residues suggesting variance in their mode of action. The PS interacting residues comprised of positively charged as well as hydrophobic and polar amino acids and their antifungal nature was evident by low binding energies (-4.7 to -2.8 kcal mol^{-1}) representing stronger ligand interactions. Defensins are known to bind and interact with phospholipids that are integral components of the fungal cell membrane supporting pore formation and membrane disruption (Poon et al. 2014). PS, a predominant anionic species represents up to 20% of the surface lipids, provide a binding site for cationic defensin residues resulting in an altered membrane charge (Yeung et al. 2008) where increased Ca^{2+} influx that might lead to PS externalization resulting in apoptosis-like condition (Cools et al. 2017). The superimposition studies also indicated a possible interaction of CaDEF1.1b with glucosylceramide (GluCer), a glycosphingolipid that facilitates the insertion of defensin peptides in the fungal plasma membrane arresting its growth (Thevissen et al. 2004; Nimrichter and Rodrigues 2011) suggesting its interaction with fungal GluCer that consequently inhibited its growth (Thevissen et al. 2010; Thevissen et al. 2007).

Conclusion

Our study is the first to identify *DEF* and *DEFL* genes in chickpea that are important components of host-plant resistance against its major pathogens. The in silico data strengthens our observations on the putative interaction of *CaDEF1.1b, CaDEF2.5a, CaDEF3, CaDEF5*, and *CaDEFL2* with chickpea pathogens, contributing to innate immunity. This study has generated significant knowledge that newer gene expression/editing tools have the potential to provide new opportunities for crop improvement with durable resistance to fungal pathogens in cultivated chickpea. Nevertheless, future studies on investigating antifungal properties of the selected defensin genes in terms of their modes of action including morphogenic and/or non-morphogenic nature remain to be elucidated to fully determine the range of pathogens that are inhibited by these antimicrobial proteins and for their possible deployment in other crops for enhanced disease resistance.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00344-022-10811-1.

Acknowledgements All authors express sincere thanks to these funding agencies for providing financial assistance, support for conducting research and other activities at ICRISAT. ICRISAT is a part of CGIAR consortium. Authors also express their sincere thanks to, Mr. Rameshwar Telangare and Mr. B Pradeep Reddy. AP acknowledges DST INSPIRE fellowship received from Department of Science and Technology, Government of India.

Author Contributions Conceptualization: RBN, PBM, and PSR; Methodology: RBN, PSR, AP, RY, AT, YR, JB, and VS; Formal Analysis and Data Curation: RBN, RY, AP, JB, YR, PSR, and VS; Original Draft Preparation: RBN, AP, and RY; Review, Editing and Supervision: PSR, PBM, and MS. All authors have read and agreed to the published version of the manuscript.

Data Availability The data presented in this study are available in the supplementary materials.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Aerts AM, François IEJA, Cammue BPA, Thevissen K (2008) The mode of antifungal action of plant, insect and human defensins. Cell Mol Life Sci 65:2069–2079

Andam A, Azizi A, Majdi M, Abdolahzadeh J (2020) Comparative expression profile of some putative resistance genes of chickpea genotypes in response to ascomycete fungus *Ascochyta rabiei* (Pass). Labr Rev Bras Bot 43(1):123–130

Anuradha T, Divya K, Jami SK, Kirti PB (2008) Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens. Plant Cell Rep 27:1777–1786

Benko-Iseppon AM, Winter P, Huettel B, Staginnus C, Muehlbauer FJ, Kahl G (2003) Molecular markers closely linked to *Fusarium* resistance genes in chickpea show significant alignments to pathogenesis-related genes located on *Arabidopsis* chromosomes 1 and 5. TAG Theor Appl Genet 107:379–386

Bohlmann H, Broekaert W (1994) The role of thionins in plant protection. Crit Rev Plant Sci 13:1–16

Cannon SB, Mitra A, Baumgarten A, Young ND, May G (2004) The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. BMC Plant Biol 4:10

Chang WC, Lee TY, Huang HD, Huang HY, Pan RL (2008) PlantPAN: plant promoter analysis navigator, for identifying combinatorial cis-regulatory elements with distance constraint in plant gene groups. BMC Genomics 9:561

Cools TL, Struyfs C, Cammue BP, Thevissen K (2017) Antifungal plant defensins: increased insight in their mode of action as a basis for their use to combat fungal infections. Future Microbiol 12:441–454

Furger A, O'Sullivan JM, Binnie A, Lee BA, Proudfoot NJ (2002) Promoter proximal splice sites enhance transcription. Genes Dev 16:2792–2799

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CM (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18:1307–1310

García R, Botet J, Rodríguez-Peña JM, Bermejo C, Ribas JC, Revuelta JL, Nombela C, Arroyo J (2015) Genomic profiling of fungal cell wall-interfering compounds: identification of a common gene signature. BMC Genomics 16:683

Graham MA, Silverstein KAT, Cannon SB, VandenBosch KA (2004) Computational identification and characterization of novel genes from legumes. Plant Physiol 135:1179–1197

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27:297–300

Hollox EJ, Armour JAL, Barber JCK (2003) Extensive normal copy number variation of a β-defensin antimicrobial-gene cluster. Am J Hum Genet 73:591–600

Innan H, Kondrashov F (2010) The evolution of gene duplications: classifying and distinguishing between models. Nat Rev Genet 11:4

Iqbal A, Khan RS, Shehryar K, Imran A, Ali F, Attia S, Shah S, Mii M (2019) Antimicrobial peptides as effective tools for enhanced disease resistance in plants. Plant Cell, Tissue Organ Cult (PCTOC) 139(1):1–15

Islam A (2008) Preliminary risk assessment of a novel antifungal defensin peptide from chickpea (*Cicer arietinum* L.). Appl Biosaf 13(4):222–230

Jain M, Misra G, Patel RK, Priya P, Jhanwar S, Khan AW, Shah N, Singh VK, Garg R, Jeena G, Yadav M (2013) A draft genome sequence of the pulse crop chickpea (*Cicer arietinum* L.). Plant J 74:715–729

Jha S, Tank HG, Prasad BD, Chattoo BB (2009) Expression of Dm-AMP1 in rice confers resistance to *Magnaporthe oryzae* and *Rhizoctonia solani*. Transgenic Res 18:59–69

Jukanti AK, Gaur PM, Gowda CLL, Chibbar RN (2012) Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br J Nutr 108(S1):S11–S26

Kaur J, Sagaram US, Shah D (2011) Can plant defensins be used to engineer durable commercially useful fungal resistance in crop plants? Fungal Biol Rev 25:128–135

Kong K, Ntui VO, Makabe S, Khan RS, Mii M, Nakamura I (2014) Transgenic tobacco and tomato plants expressing Wasabi defensin genes driven by root-specific LjNRT2 and AtNRT2.1 promoters confer resistance against *Fusarium* oxysporum. Plant Biotechnol 31:89–96

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA (2009) Circos: an information aesthetic for comparative genomics. Genome Res 19:1639–1645

Kwek KY, Murphy S, Furger A, Thomas B, O'Gorman W, Kimura H, Proudfoot NJ, Akoulitchev A (2002) U1 snRNA associates with TFIIH and regulates transcriptional initiation. Nat Struct Biol 9:800

Lacerda A, Vasconcelos ÉAR, Pelegrini PB, Grossi-de-Sa MF (2014) Antifungal defensins and their role in plant defense. Front Microbiol 5:116

Lay F, Anderson M (2005) Defensins - Components of the innate immune system in plants. Curr Protein Pept Sci 6:85–101

Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P (2002) Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327

Li H, Rodda M, Gnanasambandam A, Aftab M, Redden R, Hobson K, Rosewarne G, Materne M, Kaur S, Slater AT (2015) Breeding for biotic stress resistance in chickpea: progress and prospects. Euphytica 204:257–288

Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. Science 290:1151–1155

Mani-López E, Palou E, López-Malo A (2021) Legume proteins, peptides, water extracts, and crude protein extracts as antifungals for food applications. Trends Food Sci Technol 112:16–24

Matsuzaki K (1999) Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochim Biophys Acta—Biomembr 1462:1–10

Matsuzaki K (2009) Control of cell selectivity of antimicrobial peptides. Biochim Biophys Acta—Biomembr 1788:1687–1692

Maxwell A, Morrison G, Dorin J (2003) Rapid sequence divergence in mammalian β-defensins by adaptive evolution. Mol Immunol 40:413–421

Mezey JG, Nuzhdin SV, Ye F, Jones CD (2008) Coordinated evolution of co-expressed gene clusters in the *Drosophila* transcriptome. BMC Evol Biol 8:2

Mirouze M, Sels J, Richard O, Czernic P, Loubet S, Jacquier A, François IE, Cammue B, Lebrun M, Berthomieu P, Marquès LA (2006) Putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance. Plant J 47:329–342

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem 30:2785–2791

Nguyen MN, Madhusudhan MS (2011) Biological insights from topology independent comparison of protein 3D structures. Nucleic Acids Res 39:e94–e94

Nimrichter L, Rodrigues ML (2011) Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials. Front Microbiol 2:212

Oliver B, Parisi M, Clark D (2002) Gene expression neighborhoods. J Biol 1:4

Pieper U, Eswar N, Davis FP, Braberg H, Madhusudhan MS, Rossi A, Marti-Renom M, Karchin R, Webb BM, Eramian D, Shen MY (2011) ModBase, a database of annotated comparative protein structure models, and associated resources. Nucleic Acids Res 39:D465–D474

Poon IK, Baxter AA, Lay FT, Mills GD, Adda CG, Payne JA, Phan TK, Ryan GF, White JA, Veneer PK, Van der Weerden NL (2014) Phosphoinositide-mediated oligomerization of a defensin induces cell lysis. Elife 3:p.e1808

Proudfoot NJ, Furger A, Dye MJ (2002) Integrating mRNA processing with transcription. Cell 108:501–512

Reddy DS, Bhatnagar-Mathur P, Reddy PS, Cindhuri KS, Ganesh AS, Sharma KK (2016) Identification and validation of reference genes and their impact on normalized gene expression studies across cultivated and wild *Cicer* species. PLoS ONE 11:2

Rose AB (2008) Intron-mediated regulation of gene expression. In: Reddy A.S.N. & Golokvin M. (ed.) Nuclear pre-mRNA

processing in plants: Curr Top Microbiol Immunol 326, (), 277–290 (Springer)
Rotem S, Mor A (2009) Antimicrobial peptide mimics for improved therapeutic properties. Biochim Biophys Acta—Biomembr 1788:1582–1592
Sagaram US, El-Mounadi K, Buchko GW, Berg HR, Kaur J, Pandurangi RS, Smith TJ, Shah DM (2013) Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: identification of an RGFRRR motif governing fungal cell entry. PLoS One 8:e82485
Semple CA, Rolfe M, Dorin JR (2003) Duplication and selection in the evolution of primate β-defensin genes. Genome Biol 4:R31
Sharma M, Pande S (2013) Unravelling effects of temperature and soil moisture stress response on development of dry root rot [*Rhizoctonia bataticola* (Taub.)] Butler in chickpea. Am J Plant Sci 4:584–589
Sharma M, Pande S, Mangala UN, Tripathi S, Gaur PM (2010) Genetic resistance in desi and kabuli chickpea lines to Fusarium wilt caused by *Fusarium* oxysporum f. sp. ciceris. Ind J Plant Prot 38:57–62
Sharma M, Ghosh R, Tarafdar A, Telangre R (2014) An efficient method for zoospore production, infection, and real-time quantification of *Phytophthora cajani* causing Phytophthora blight disease in pigeonpea under elevated atmospheric CO2. BMC Plant Biol 15(1):1–12
Silverstein KAT, Graham MA, Paape TD, VandenBosch KA (2005) Genome organization of more than 300 defensin-like genes in *Arabidopsis*. Plant Physiol 138:600–610
Takeuchi H, Higashiyama T, Dumas C, Cock J, Kawamukai MA (2010) species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*. PLoS Biol 10:e1001449
Tarafdar A, Rani TS, Chandran USS, Ghosh R, Chobe DR, Sharma M (2018) Exploring combined effect of abiotic (soil moisture) and biotic (*Sclerotium rolfsii* Sacc.) stress on collar rot development in chickpea. Front Plant Sci 9:1154
Thevissen K, Warnecke DC, François IE, Leipelt M, Heinz E, Ott C, Zähringer U, Thomma BP, Ferket KK, Cammue BP (2004) Defensins from insects and plants interact with fungal glucosylceramides. J Biol Chem 279:3900–3905
Thevissen K, Kristensen HH, Thomma BPHJ, Cammue BPA, François IEJA (2007) Therapeutic potential of antifungal plant and insect defensins. Drug Discov Today 12:966–971
Thevissen K, de Mello TP, Xu D, Blankenship J, Vandenbosch D, Idkowiak-Baldys J, Govaert G, Bink A, Rozental S, De Groot PW, Davis TR (2010) The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in *Candida albicans*. Mol Microbiol 84:166–180
Thomma B, Cammue B, Thevissen K (2002) Plant defensins. Planta 216:193–202
Trott O, Olson AJ (2009) AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 31:455–461
Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35:W71–W74
Varshney RK, Song C, Saxena RK, Azam S, Yu S, Sharpe AG, Cannon S, Baek J, Rosen BD, Tar'an B, Millan T (2013) Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. Nat Biotechnol 31:240–246
Vriens K, Cammue B, Thevissen K (2014) Antifungal plant defensins: mechanisms of action and production. Molecules 19:12280–12303
Wang S, Rao P, Ye X (2009) Isolation and biochemical characterization of a novel leguminous defense peptide with antifungal and antiproliferative potency. Appl Microbiol Biotechnol 82(1):79–86
Wijaya R, Neumann GM, Condron R, Hughes AB, Polya GM (2000) Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. Plant Sci 159:243–255
Williams EJB, Bowles DJ (2004) Coexpression of neighboring genes in the genome of *Arabidopsis thaliana*. Genome Res 14:1060–1067
Xiao Y, Hughes AL, Ando J, Matsuda Y, Cheng JF, Skinner-Noble D, Zhang GA (2004) genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics 5:56
Yan J, Yuan SS, Jiang LL, Ye XJ, Ng TB, Wu ZJ (2015) Plant antifungal proteins and their applications in agriculture. Appl Microbiol Biotechnol 99(12):4961–4981
Yang X, Wang J (2016) Genome-wide analysis of NBS-LRR genes in *Sorghum* genome revealed several events contributing to NBS-LRR gene evolution in grass species. Evol Bioinform 12:9–21
Yang D, Chertov O, Oppenheim JJ (2001) Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). J Leukoc Biol 69:691–697
Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S (2008) Membrane phosphatidylserine regulates surface charge and protein localization. Science 319:210–213